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Prevention of Acute Allograft Rejection by Antibody Targeting of TIRC7, a Novel T Cell Membrane Protein

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Summary

A novel 75 kDa membrane protein, TIRC7, is described that exhibits a central role in T cell activation in vitro and in vivo. Modulation of TIRC7-mediated signals with specific anti-TIRC7 antibodies in vitro efficiently prevents human T cell proliferation and IL-2 secretion. Moreover, anti-TIRC7 antibodies specifically inhibit type 1 subset specific IFN-γ expression but spare the type 2 cytokine IL-4. Diminished proliferation but not IFN-γ secretion is reversible by exogenous rIL-2. An anti-TIRC7 antibody that cross-reacts with the 75 kDa rat homolog exhibits inhibition of rat alloimmune response in vitro and significantly prolongs kidney allograft survival in vivo. Targeting of TIRC7 may provide a novel therapeutic approach for modulation of the immune response.

Introduction

T cell activation is a serial process involving multiple signaling pathways and sequential changes in gene expression resulting in differentiation of T cells into distinct subpopulations, i.e., Th1 and Th2, which are distinguishable by their pattern of cytokine production and characterize the mode of the cellular immune response (Abbas et al., 1996; Crabtree, 1989). The T cell response is initiated by the interaction of the antigen-specific T cell receptor (TCR) with peptide presented by major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs). Additional signals are provided by a network of receptor–ligand interactions mediated by a number of membrane proteins such as CD28/CTLA4 and B7, CD40/CD40L, LFA-1 and ICAM-1 (Schwartz, 1992; Linsley and Ledbetter, 1993; Xu et al., 1994; Lenschow et al., 1996; Bachmann et al., 1997), collectively called costimulatory signals (Walunas et al., 1994; Perez et al., 1997). These membrane proteins can alter T cell activation in distinct ways (Bachmann et al., 1997) and regulate the immune response by the integration of positive and negative signals provided by these molecules (Bluestone, 1995; Perez et al., 1997).

Many of the agents that are effective in modulating the cellular immune response either interfere with the T cell receptor (Cosimi et al., 1981), block costimulatory signaling (Linsley et al., 1992; Turka et al., 1992; Blazar et al., 1996; Larsen et al., 1996; Kirk et al., 1997), or inhibit intracellular activation signals downstream from these primary cell membrane triggers (Schreiber and Crabtree, 1992). Therapeutic prevention of T cell activation in organ transplantation and autoimmune diseases presently relies on panimmunosuppressive drugs interfering with downstream intracellular events. Specific modulation of the T cell response remains a long-standing goal in immunological research.

The present study was conducted in order to identify novel transcripts that are induced early after T cell stimulation with alloantigen, as products of such transcripts are likely to play an important role in the activation process or its regulation and might be targets for specific modulation. We describe a differentially expressed T cell mRNA, termed *TIRC7*, that is transiently upregulated in early T cell stimulation with alloantigen. The transcript encodes a membrane protein that plays an essential role in T cell activation. The biological significance of the protein is underscored by the fact that targeting of TIRC7 with specific antibodies significantly prolongs rat kidney allograft survival. Therefore, TIRC7 may serve as a target for in vivo modulation of cellular immune response in the context of organ transplantation and autoimmune diseases.

Results

TIRC7 Is Differentially Expressed in Alloactivated Human T Cells and Encodes a Novel Membrane Protein

To identify novel genes induced during the early stages of T cell activation in response to alloantigens, differential display RT-PCR analysis of mRNA expression was performed at time 0 and 24 hr after initiation of a human mixed lymphocyte culture (MLR). As shown in Figure 1A, a 350 bp cDNA fragment was identified that was induced at 24 hr after stimulation, and was used to identify several clones from λ-gt-10 cDNA libraries from

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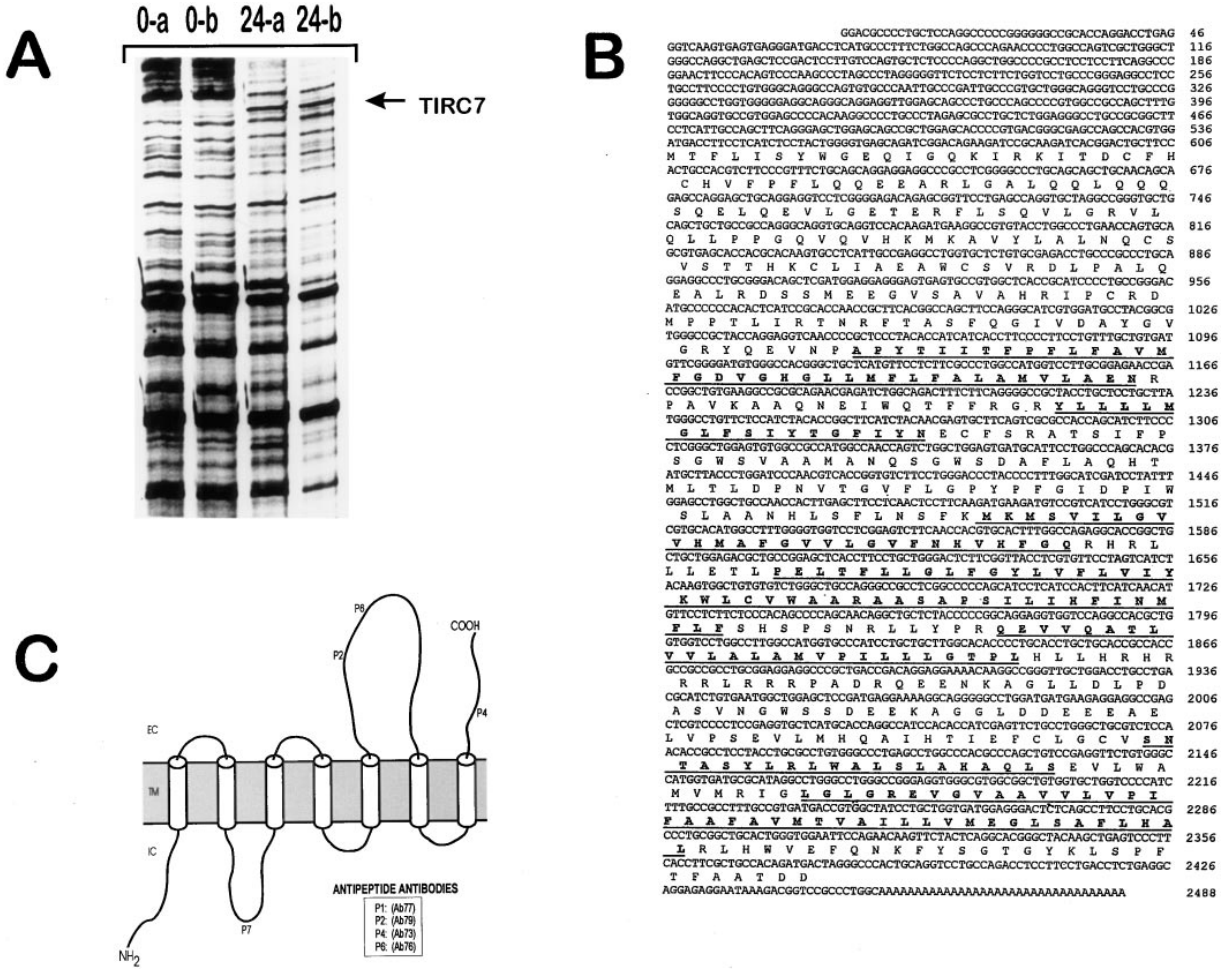

Figure 1. Identification of TIRC7 from Alloactivated T Cells

(A) Differential display identification of a 350 bp transcript upregulated 24 hr after alloactivation of human T lymphocytes. Each lane shows an mRNA expression pattern from a one-way MLR at either 0 or 24 hr after activation. Two different MLRs (a and b) exhibited similar patterns of gene expression.

(B) Nucleotide sequence of *TIRC7* cDNA. The cDNA and deduced 614 amino acid sequences of the TIRC7 transcript are shown. Predicted transmembrane regions are underlined and bold.

(C) The predicted secondary structure of TIRC7 protein contains seven transmembrane spanning domains (TM). Peptides (P1–P7) synthesized according to sequences in the putative intracellular amino terminus (NH3), extracellular carboxy terminus (COOH), and the largest intracellular (IC) and extracellular (EC) loop were used to raise rabbit anti-TIRC7 polyclonal antibodies. Anti-TIRC7 antibodies with immune modulatory effects are given in the box.

human T cells. Sequence analysis revealed a 2488 bp cDNA that was designated as *TIRC7* (*T* cell *i*mmune *r*esponse *c*DNA *7*; GenBank accession number: AF025374), containing an open reading frame of 1842 nt and predicting a protein length of 614 amino acids (Figure 1B). Hydrophobicity analysis of the protein sequence revealed seven hydrophobic domains, compatible with transmembrane spanning domains. The N terminus of TIRC7 lacks a consensus signal peptide sequence and, as it is followed by seven hydrophobic domains, the protein has a predicted topology of an intracellular N terminus and extracellularly oriented C domain (Figure 1C). TIRC7 contains multiple putative sites of posttranslational modification including phosphorylation sites for PKC (at amino acids 58, 98, and 148) and PKA (at amino acid 21), as well as N-linked glycosylation sites (at amino acid 267 and 287). No amino acid homology was found with any proteins known to be involved in T cell activation. TIRC7 does share amino acid homology (12%–71%) with several proteins reported as putative subunits of the vacuolar proton pump H^+-ATPase (VPP) in a variety of species (Bowman et al., 1988; Lee et al., 1990; Perin et al., 1991; Manolson et al., 1992, 1994; Peng et al., 1994; Solioz and Davies, 1994; Li et al., 1996). Analysis of the complete genomic DNA organization of *TIRC7* revealed that *TIRC7* and a recently reported human cDNA, named *OC116* (Li et al., 1996), are alternatively spliced transcripts of the same gene (data not shown). The function of *OC116* is so far unknown and the 2640 nt mRNA, encoding an 829-residue protein, was demonstrated to be exclusively expressed in human osteoclastoma cells. The regions of strongest homology between TIRC7 and these putative VPPs are predominantly in their predicted transmembrane domains and the C

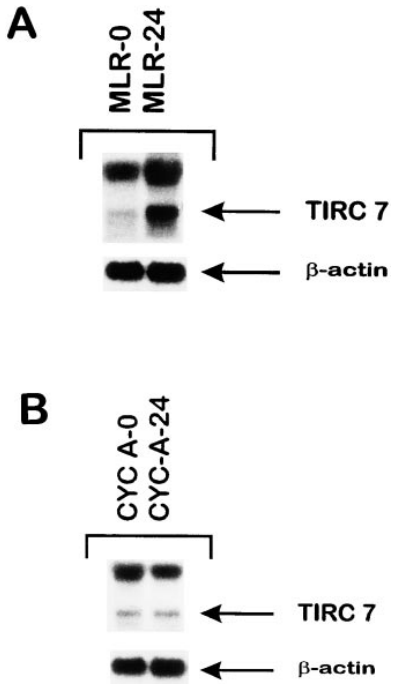

Figure 2. *TIRC7* mRNA Is Upregulated after Allostimulation of Human T Cells

(A) *TIRC7* mRNA expression is upregulated in allostimulated human T cells. MLR-0 and MLR-24 indicate time points 0 hr and 24 hr, respectively, after coincubation of allogenic responder and stimulator lymphocytes.

(B) *TIRC7* mRNA upregulation in MLR (24 hr) is prevented by Cyclosporin A (Cyc A).

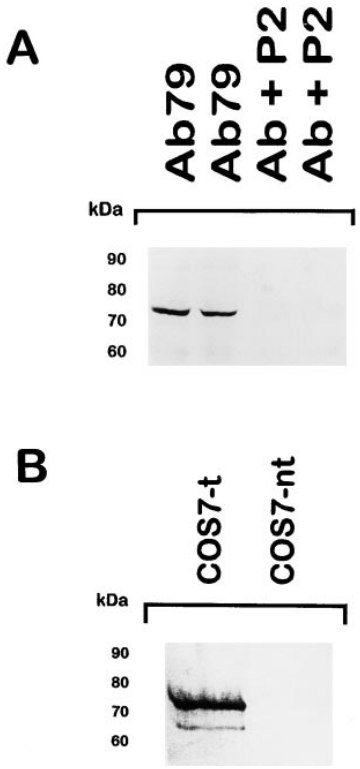

Figure 3. Identification of the TIRC7 Protein

(A) A single 75 kDa protein is detected by anti-TIRC7 antibodies (Ab79 is shown) predominantly in membrane protein extracts of human lymphocytes. Binding of Ab79 to TIRC7 is abolished in the presence of the respective peptide P2 (Ab+P2).

(B) The same single 75 kDa protein is also detected by an anti-*c-myc* antibody in membrane preparations of COS-7 cells transiently transfected with a c-*myc*-tagged TIRC7 expression vector (COS7-t) but not in untransfected COS-7 cells (COS7-nt).

termini. Thus, TIRC7 belongs to a larger family of structurally related membrane proteins whose functions have not been clearly elucidated.

To determine the expression kinetics of *TIRC7*, Northern blot analysis of total RNA from alloantigen-activated lymphocytes was performed. A *TIRC7*-specific cDNA probe detected the expected 2.5 kb transcript as well as an additional 4 kb mRNA of unknown origin (Figure 2A). Alloactivation of T cells resulted in a 20-fold upregulation of *TIRC7* expression at 24 hr (Figure 2A). *TIRC7* expression was transient with no increase at 1 hr, peak expression at 24 hr, and a return to baseline at 72 hr (data not shown). To ensure that the upregulation of *TIRC7* occurred in the responder T cell population, an additional MLR was performed using stimulators depleted of T cells and *TIRC7* mRNA expression was found to be increased in responder T cells 24 hr after coculture (data not shown).

Cyclosporine A (Cyc A), an inhibitor of the calcineurin-dependent T cell activation pathways, blocked the induction of *TIRC7* in an MLR (Figure 2B). Not shown, exogenous high-dose IL-2 was a potent inducer of *TIRC7* expression, whereas a modest increase in *TIRC7* expression was observed with *Staphylococcus aureus* enterotoxin B (SEB) or OKT3-MAb stimulation after 24 hr, though OKT3-MAb increased *TIRC7* expression after 48 hr to a similar level as that induced by alloantigen. In contrast, neither concanavalin A (ConA) nor phytohemagglutinin (PHA) increased *TIRC7* expression at 24 or 48 hr.

Northern analysis revealed that *TIRC7* is almost exclusively expressed in immune tissues and exhibits high levels of mRNA expression in spleen, lymph nodes, peripheral blood, and appendix, whereas lower levels of expression are observed in bone marrow, fetal liver, and thymus, respectively. *TIRC7* was also detected in $CD4^+$ and $CD8^+$ lymphocytes but not in EBV-transformed primary B cells, Burkitt's lymphoma cells, EBV-infected Burkitt's lymphoma cells, and resting or activated Jurkat cells (data not shown).

In Western blot analysis, a single protein of approximately 75 kDa molecular mass was detected predominantly in membrane extracts of human lymphocytes (Figure 3A). The same protein was found in membrane preparations from CHO cells (data not shown) and COS-7 cells (Figure 3B) stably and transiently transfected with a c-*myc*-tagged TIRC7 expression vector, respectively. TIRC7 localization to the cell membrane was confirmed by confocal microscopy in human lymphocytes (Figures 4A and 4B) as well as in stably transfected CHO cells (data not shown). Small amounts of TIRC7 protein was also detected in the intracellular compartment of the lymphocytes suggesting that preformed protein may exist in an endosomal compartment that is transported to the cell surface upon T cell activation.

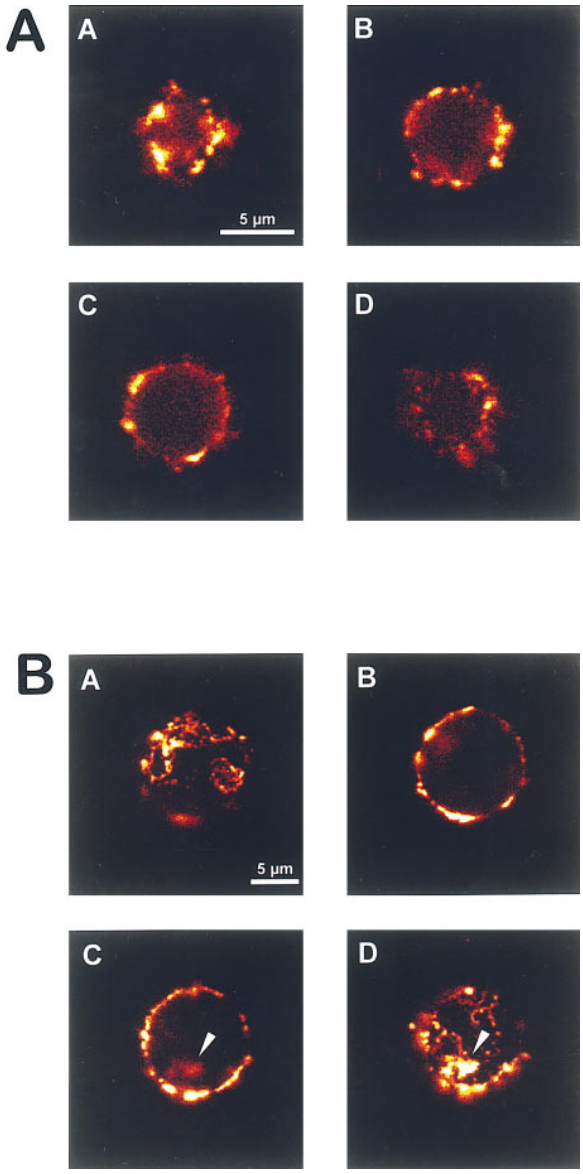

Figure 4. Cellular Localization of TIRC7 Protein

Confocal microscopy shows the localization of TIRC7 protein in the cell membrane (parts A and B) (yellow fluorescence) and intracellular pools (part B, panels C and D; arrowheads) of human lymphocytes. T cells were fixed (part A) and additionally permeabilized (part B). Cells were stained for TIRC7 protein with Ab79 as shown in serial optical sections taken at 1.5 μm (part A) and 1.9 μm (part B) intervals beginning at the surface and continuing through horizontal sections of the T cell (parts A and B, panels A–D). No staining of TIRC7 protein was detected when the Ab79 was neutralized by addition of the respective peptide (not shown).

TIRC7 Mediates an Essential Signal during Early Events of T Cell Activation

The functional significance of a number of proteins required in T cell activation has been determined by modulation of their signaling by targeting with specific antibodies. To examine whether antibodies directed against TIRC7 could alter the T cell proliferative response, seven synthetic peptides representing different domains of the TIRC7 protein (P1–P7; 12-mers) were used to produce polyclonal rabbit antipeptide antibodies (Figure 1C). As shown in Figure 5A, three of the antibodies Ab73, Ab76, and Ab79, which were directed against the extracellularly located domains, respectively, were found to strongly inhibit the proliferation of alloactivated T cells. In contrast, two antibodies directed against extracellular epitopes and two antibodies recognizing intracellular domains of TIRC7 did not exhibit immune modulatory effects on human T cell activation. The anti-TIRC7 antibodies Ab73, Ab76, and Ab79, inhibited T cell proliferation in a dose-dependent manner (data not shown). Addition of the respective TIRC7 peptides, but not peptides unrelated to TIRC7, diminished inhibition indicating the specific neutralization of the antibody. The antibodies had no effect when added 24–72 hr after initiating the MLR, suggesting that the TIRC7-mediated signal was specific for an early event in the T cell response and proliferation inhibition was not due to a toxic effect of the antibodies. Moreover, no evidence for toxicity was observed by the antibodies as examined by trypan blue staining. The same three anti-TIRC7 antibodies also caused efficient inhibition of T cell activation induced by ConA, PHA, and OKT3-MAb, respectively (data not shown).

Membrane proteins associated with T cell activation are often involved in ligand–receptor interactions that can be blocked by exogenous soluble protein, as has been demonstrated by blocking of CD28/B7 interaction with the soluble protein CTLA4Ig (Linsley et al., 1992). In vitro–translated TIRC7 protein was therefore tested for its ability to inhibit the MLR by adding it to MLR cultures at time 0 (Figure 5B). Two other in vitro–translated seven-transmembrane spanning proteins, Rantes receptor (CCR1) and Leukotriene B4 receptor, as well as a nonmembrane protein (unpublished), served as negative controls. Only exogenous TIRC7 protein significantly suppressed the proliferation of alloreactive T cells in a dose-dependent manner. In control experiments, no inhibition was observed using other in vitro–translated membrane or nonmembrane proteins (Figure 5B).

Targeting of the TIRC7-Mediated Signal Inhibits Type 1–Specific Cytokine Expression

To further differentiate whether signals mediated by TIRC7 differentially affect T cell subsets, human T cells were challenged with either OKT3-MAb, ConA, or PHA, and the cytokine profiles specific for type 1 and type 2 lymphocyte subsets were analyzed in the presence and absence of anti-TIRC7 antibodies. As shown in Figure 5C, a significant decrease of the type 1–specific cytokines IL-2 and IFN-γ was observed at 24 hr in all cultures of PHA-stimulated lymphocytes. Of the three stimuli tested, the IL-2 and IFN-γ downregulation occurred in ConA- and PHA-activated cultures at 24 hr, whereas the OKT3-MAb-stimulated T cells showed a significant decrease of IL-2 at 24 hr but of IFN-γ only after 48 hr (data not shown). No downregulation of IL-4 production, which is specific for type 2 T lymphocytes, was observed in any of the mitogen-activated T cells after 24 and 48 hr. Moreover, quantitative RT-PCR studies demonstrated a significant decrease of PHA-induced IL-2 mRNA expression after 24 hr in the presence of anti-TIRC7 antibody

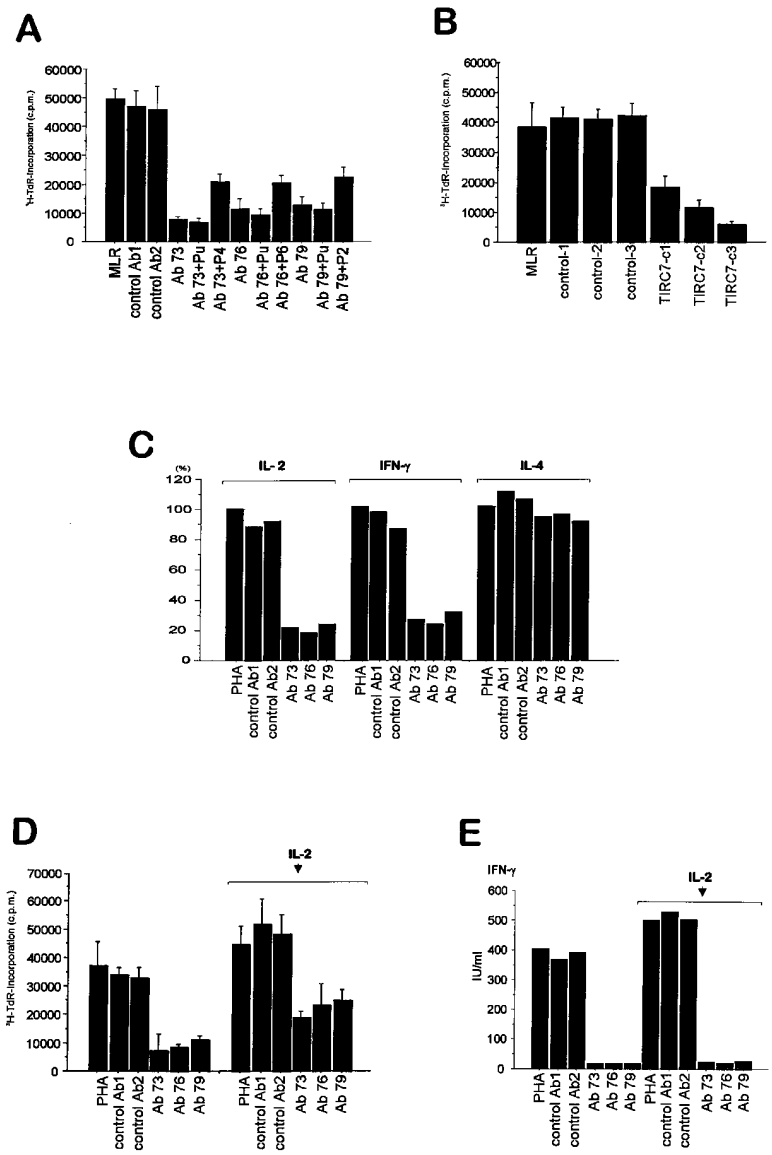

Figure 5. Anti-TIRC7 Antibodies Inhibit T Cell Proliferation and Type 1–Specific Cytokine Production

In (A) and (C)–(E), control antibody 1 (control Ab1) represents preimmune serum and control antibody 2 (control Ab2) represents Ab77, respectively.

(A) Anti-TIRC7 antibodies (Ab73, Ab76, Ab79) directed against extracellularly located TIRC7 peptides (P4, P6, P2) inhibit proliferation in alloantigen-stimulated T cells as determined by [^{3}H]-thymidine incorporation. Inhibition was diminished with their respective TIRC7 peptides, whereas no changes of anti-TIRC7 antibody mediated inhibition was observed by adding TIRC7 unrelated peptides (pu) to each of the antibodies. Proliferation in an MLR is displayed as positive control. Proliferation was not affected by control antibody directed against putative intracellular domain of TIRC7 protein (Ab 77). Each bar represents mean ± SD from three independent experiments.

(B) Inhibition of proliferation by exogenous TIRC7 protein. In a one-way MLR in vitro–translated TIRC7 protein inhibited proliferation in a dose-dependent manner (TIRC7-c1 = 30 ng/ml protein; TIRC7-c2 = 50 ng/ml protein and TIRC7-c3 = 70 ng/ml protein). Two other seven-membrane TIRC7 unrelated proteins, control-1 (Rantes receptor CCR1), control-2 (Leukotriene B receptor) or control-3 (nonreceptor protein-c7) were used as negative controls. Shown is a concentration of 70 ng/ml for all negative control experiments. Each bar represents the mean ± SD of three independent experiments.

(C) Anti-TIRC7 antibodies inhibit Th1-specific cytokine expression. PHA-stimulated human lymphocytes were coincubated with Ab73, Ab76, and Ab79, respectively. Supernatants of mitogen-stimulated cultures were taken at 24 hr, and cytokine expression in the supernatants was determined by ELISA. Each bar represents the mean from three independent experiments (100% = 661 pg/ml for IL-2, 221 UI/ml for IFN-γ, and 27 pg/ml for IL-4).

(D) Exogenous rIL-2 restores the diminished T cell proliferation by antibody targeting of TIRC7 protein. Anti-TIRC7 antibody mediated inhibition of proliferation of PHA-activated T cells that is reconstituted by exogenous rIL-2 (arrow). Each bar represents the mean ± SD from four independent experiments.

(E) IFN-γ is decreased in the PHA-activated cultures in the presence of anti-TIRC7 antibodies within the first 24 hr, and it remains decreased when exogenous rIL-2 is added (arrow) to the culture for a second 24 hr period. Each bar represents the mean of three independent experiments.

(22.8 AU/μl cDNA in PHA-stimulated lymphocyte controls, 33.5 AU/μl cDNA in control antibody cultures, and 4.6 AU/μl cDNA in PHA-activated lymphocytes in the presence of anti-TIRC7 antibody Ab73; $p < 0.01$). These results suggest that TIRC7 targeting affected T cell activation signaling pathways involving the IL-2 mRNA expression.

Remarkably, exogenous addition of recombinant IL-2 to mitogen-activated cultures incubated with anti-TIRC7 antibodies restored, at least partially, the diminished T cell proliferation. The restoration of T cell proliferation by rIL-2 was dependent on the concentration of anti-TIRC7 antibodies. At high concentrations (1:500 dilution) the inhibition of T cell proliferation was only partially restored in the presence of rIL-2 (Figure 5D), whereas complete reversion of the inhibition was achieved when anti-TIRC7 antibodies were present at lower concentration (1:1000 dilution; data not shown).

To further analyze whether the specific inhibition of the type 1 cytokine IFN-γ was indirectly due to a lack of IL-2 expression, human peripheral lymphocytes were challenged with PHA for 24 hr in the presence of anti-TIRC7 antibodies. rIL-2 was added 24 hr after culture initiation for another 24 hr period. IFN-γ concentration was determined prior to and 24 hr after IL-2 incubation. Although diminished T cell proliferation was partially restored (Figure 5D), the IFN-γ secretion remained continuously suppressed in the presence of rIL-2 for the second 24 hr period (Figure 5E), indicating that anti-TIRC7 antibody mediated a stable inhibition of type 1

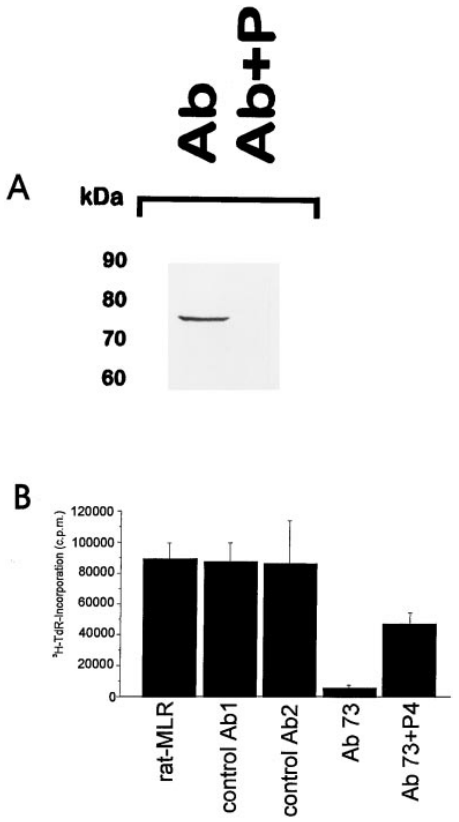

Figure 6. Anti-Human TIRC7 Antibody (Ab 73) Cross-Reacts with a Rat TIRC7 Homolog

(A) Anti-TIRC7 antibody (Ab) recognizes a 75 kDa protein in the protein lysates from rat lymphocytes, and the reaction is diminished by addition of the appropriate peptide to the antibody (Ab+P).

(B) Anti-TIRC7 antibody blocks significantly the MLR from Lewis rat T cells (responder) and spleen cells from Wistar Furth (stimulator) rats. Inhibition was diminished by adding the respective peptide to the reaction.

response that was not exclusively due to the lack of IL-2. Similar results were obtained when anti-TIRC7 antibodies were used at lower concentration in the cultures (data not shown).

Antibody Targeting of TIRC7 Significantly Prolongs Renal Allograft Survival In Vivo

The effect of modulating the TIRC7-mediated signal was studied in an animal model featuring kidney transplantation from Wistar Furth to Lewis rats. In initial experiments, anti-human anti-TIRC7 antibody Ab73, which recognized a single protein of 75 kDa in Western blot analysis from rat lymphocyte lysates (Figure 6A), was selected for its ability to inhibit the proliferation of Lewis rat lymphocytes stimulated with irradiated Wistar Furth rat lymphocytes in vitro. Ab73 was shown to profoundly block rat T cell proliferation (Figure 6B). In kidney transplant experiments, animals either remained untreated ($n = 7$), received preimmune rabbit serum ($n = 7$) or anti-TIRC7 antibody recognizing a putative intracellular domain of the TIRC7 protein (Ab 77) ($n = 7$), or were treated with anti-TIRC7 antibody Ab73 ($n = 7$), 2 hr before, directly after, and on days 1, 2, 4, and 6 after transplantation (Figure 7A). No side effects except for transient mild diarrhea were observed in the anti-TIRC7 antibody (Ab73) treated group. Quantification of rat CD3-, CD8-, and CD4-positive T cells flow-cytometrically measured at 24 hr, day 2, and day 10 after the anti-TIRC7 antibody treatment did not reveal a depletion of the peripheral T cells (data not shown). Anti-TIRC7 (Ab73) antibody significantly prolonged the graft survival time of treated animals ($p < 0.001$) (Figure 7A). Six of seven allografts of the Ab73-treated animals remained functional for more than 40 days after completion of antibody administration. One animal treated with anti-TIRC7 antibody (Ab73) died at day 21, three animals around day 45, and three animals around day 80. In contrast, all animals in control groups died of renal failure by day 7 to 9 after transplantation. Histological examination of kidney grafts from two additional Ab73-treated animals sacrificed at day 7 posttransplantation demonstrated very mild lymphocytic infiltration but no signs of tissue necrosis (Figure 7B). In contrast, kidney grafts from control animals displayed remarkable evidence of acute graft rejection including diffuse mononuclear cell infiltrates as well as extensive areas of necrosis (Figure 7C).

Discussion

TIRC7 represents a novel protein that plays an essential role in T cell activation. Early after stimulation of the T cell receptor the level of *TIRC7* mRNA is transiently increased. Upregulation of *TIRC7* is also observed after incubation of T cells with exogenous rIL-2. The blockade of *TIRC7* mRNA upregulation is achieved with cyclosporine A, indicating that TIRC7 expression is via calcineurin signaling pathway. The pattern of tissue expression suggests that TIRC7 is a product of mature lymphoid cells, as TIRC7 is expressed in all lymphoid tissues with low expression only in thymus, bone marrow, and fetal liver. The TIRC7 protein is predominantly expressed on the cell membrane, consistent with a target for an external ligand. The seven-transmembrane domain structure predicts three extracellular loops and an extracellularly oriented carboxy terminus. Anti-TIRC7 antibodies directed against the extracellular domains, but not those recognizing predicted intracellular domains of the protein, are able to efficiently suppress the proliferation of T cells in response to alloactivation or to mitogens. The inhibitory effect of anti-TIRC7 antibodies on T cells induced by a variety of different stimuli suggests that TIRC7 plays a central role in T cell activation.

Interestingly, the inhibitory effect of anti-TIRC7 antibodies is only observed when added at the initiation of the T cell activation process, even before *TIRC7* mRNA upregulation occurs suggesting that the constitutively low-level expressed TIRC7 protein on resting T cells is sufficient to trigger essential processes required in early T cell activation.

In addition, inhibition of T cell proliferation by antibody targeting of TIRC7 may suggest the existence of a ligand specifically interacting with TIRC7. Support for this hypothesis is provided by the dose-dependent inhibition of T cell proliferation in an MLR in the presence of in

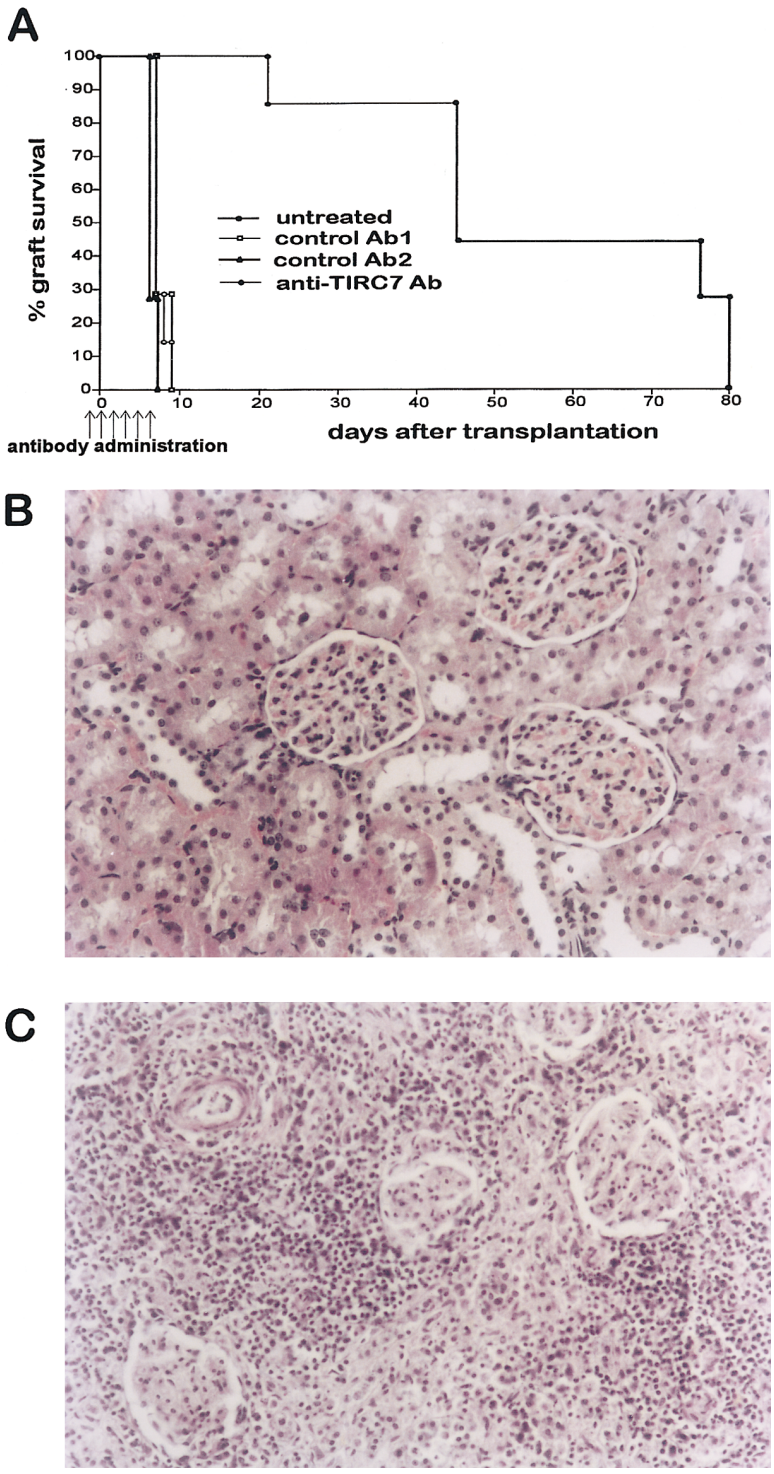

Figure 7. Anti-TIRC7 Antibody Targeting In Vivo Significantly Prolongs Allograft Survival

(A) Lewis rat recipients of Wistar Furth rat kidney allografts received either anti-TIRC7 Ab73 (n = 7), control antibody 1 (preimmune serum) (n = 7), control antibody 2 (recognizing an intracellular domain of human TIRC7 protein, Ab77) (n = 7), or no treatment (n = 7), respectively. Treatment was initiated at 2 hr prior to and immediately after transplantation, and was repeated on days 1, 2, 4, and 6 posttransplantation. Animals in control groups showed a mean survival time of 7 ± 2 days. The mean survival time in the Ab73-treated group was 56 days (range 21–80) ($p < 0.001$).

(B) Histological analysis of kidney allografts at day 7 posttransplantation. Renal allografts of two additional anti-TIRC7 antibody treated animals sacrificed at day 7 showed very mild interstitial infiltration of mononuclear cells. Tissue lesions were not identified in the allografts of these animals.

(C) Kidney allografts of rats receiving control antibodies (day 7) showed severe tissue destruction and diffuse mononuclear infiltration that was similar to histological findings in the kidney allografts of untreated animals.

vitro–translated TIRC7 protein. The lack of inhibition by other in vitro–translated seven-membrane spanning proteins used as controls (Rantes receptor CCR1, Leukotriene B4 receptor) indicates specificity of the inhibitory effect of TIRC7 protein.

TIRC7 shares 38% amino acid homology with J6B7, a protein isolated from a mouse T cell line (Lee et al., 1990). Like TIRC7, J6B7 exhibits considerable homology to the putative rat H^+-ATPase subunit VPP116 (Manolson et al., 1992). In vitro–translated J6B7 protein was demonstrated to inhibit mouse T cell proliferation in an MLR by 89%, which is comparable with the results obtained with in vitro–translated TIRC7 protein in human MLR in the present study.

Antibody targeting of TIRC7 has a selective inhibitory effect on the type 1 lymphocyte subset, as evidenced

by the inhibition of IL-2 and IFN-γ, but not IL-4, cytokine production. Although exogenous recombinant IL-2 reversed the antiproliferative effect of the anti-TIRC7 antibodies, suppression of IFN-γ secretion was not abolished, suggesting a selective induction of a stable type 1 T cell subset inhibition. These results also indicate that TIRC7 is involved in IL-2 regulation, as antibody targeting of TIRC7 was accompanied with substantial decrease of IL-2 that was also demonstrated on the transcriptional level.

Moreover, we have shown that an anti-human TIRC7 antibody cross-reacting with a rat TIRC7 homolog was able to profoundly block the rat alloimmune response both in vitro and in vivo. The polyclonal anti-human-TIRC7 protein derived rabbit antibody did not deplete peripheral T cells in rats. Confocal microscopy and flow cytometry experiments (not shown) demonstrated low density expression of TIRC7 on the surface of resting human T cells. As antibody induced depletion of peripheral T cells requires sufficient coating of the cells by the respective antibody, this fact might explain the lack of T cell depletion by anti-TIRC7 antibody despite constitutive TIRC7 expression and suggests that in rats the absence of significant kidney intragraft T cell infiltration is due to prevention of T cell alloactivation as observed in vitro.

The effects of antibody targeting of TIRC7 are quite similar to those observed by targeting of costimulatory molecules. The interruption of CD28/B7 interaction with the soluble protein CTLA4Ig or with anti-B7-1 or -B7-2 antibodies caused inhibition of T cell proliferation (Lenschow et al., 1992; Linsley et al., 1992; Sayegh et al., 1995; Larsen et al., 1996; Blazar et al., 1996). Similar effects have been observed by blocking of CD40/CD40L interaction (Larsen et al., 1996). Furthermore, it was shown that administration of CTLA4Ig in an in vivo model of kidney allograft transplantation blocked the Th1 and sparing Th2 cytokine response and prolonged graft survival (Sayegh et al., 1995). Although these similarities to our results may suggest a costimulatory function, TIRC7 does not share structural or sequence homology with any of the known T cell accessory molecules. Thus, TIRC7 may participitate in a distinct signaling pathway induced early in the course of T cell activation. This possibility is supported by recent reports that interference with pathways mediated by molecules other than the known costimulatory proteins can modulate the T cell response. For example, antibody targeting of the common leukocyte antigen CD45RB was shown to result in a prevention of graft rejection in mice (Lazarovits et al., 1996).

Further work will be needed to elucidate in detail the function of TIRC7 in T cell activation. Given the functional similarities between TIRC7 and the known T cell accessory molecules, it is likely that the structural novelty of TIRC7 will contribute to the understanding of distinct mechanisms in the T cell response. Moreover, the striking capacity of anti-TIRC7 antibody to significantly prolong allograft survival in vivo may provide a novel approach for a selective inhibition of undesired T cell activation in human organ transplantation and autoimmune diseases.

Experimental Procedures

Mixed Lymphocyte Reaction and Modulation of T Cell Activation

In conformance with institutional policies regarding human experimentation, peripheral blood lymphocytes (PBLs) were isolated from healthy human volunteers using standard Ficoll centrifugation methods and diluted into RPMI containing 10% fetal calf serum. Responder PBLs were stimulated with equal numbers of irradiated (3000 rad, 13 min) stimulator PBLs. Cells were cocultured for 24 hr in tissue flasks at an initial concentration of 10^6 cells/ml for RNA isolation. For studies on induction or inhibition of TIRC7 expression, PBLs were exposed to concanavalin A (10 μg/ml), phytohemagglutinin (PHA) (2 μg/ml), *Staphylococcus aureus* enterotoxin B (10 μg/ml), OKT3-MAb (10 μg/ml), cyclosporine A (1 μg/ml), or rIL-2 (10 U/ml). The stimulation with OKT3-MAb was carried out by immobilizing the antibody on plastic culture plates overnight at 4°C before adding the cell suspension. For RNA isolation from $CD4^+$ and $CD8^+$ human T cells, PBLs were incubated with immunomagnetic beads coated with anti-CD4 or anti-CD8 IgG and then subjected to magnetic separation.

Differential Display Reverse Transcriptase–PCR Analysis

Total RNA was isolated from MLR at 0 and 24 hr using the RNAzol B method (Tel-Test, Inc.) and differential display was performed as described previously (Kojima et al., 1996). Briefly, 2 μg of total RNA was reverse transcribed using an oligo-dT primer and 200 U MMLV reverse transcriptase (GIBCO–BRL). A 40 cycle PCR amplification with a total volume of 10 μl was performed by using 1 μg of cDNA, 10 mM dNTPs, 1.25 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 nM primer, 5 μCi ^{35}S-dATP, and 0.3 U *Taq* polymerase. The primers for the PCR amplification were: 5′-GACGGAACAGCTTC-3′ and 5′-TGCGTCTGGTTCT-3′. The PCR products were stored at 4°C and separated by electrophoresis in 6% polyacrylamide-urea gels, transferred to filter paper, dried, and autoradiographed. The differentially expressed cDNA fragment was excised from the gel, eluted, reamplifed, cloned into pBluescript SK^+ plasmid, and sequenced at the Howard Hughes Biopolymers Research Facility or the Dana Farber Cancer Institute Biopolymer Facility at Harvard Medical School. Homology searches were performed using BLAST at NCBI. Alignments were performed using Geneworks 2.1.1.

Quantitative RT-PCR analysis for IL-2 mRNA quantitation was performed by using human IL-2 control fragment (Tip Molbiol., Berlin, Germany) as described by Platzer et al., 1996.

Cloning of the Full-Length cDNA and Genomic DNA

A λ-gt-10 cDNA library (Clontech) prepared from human T cells activated for 48 hr with PHA was screened with the 350 bp *TIRC7* cDNA fragment. Briefly, plaque lifts of 1,200,000 independent cDNA clones were hybridized with a ^{32}P-labeled cDNA for 24 hr at 42°C in 40% formamide, 10% dextran sulfate, 4× SSC (1× SSC consists of 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.8× Denhardt's solution (1× Denhardt's contains of 0.02% polyvinylpyrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS), and 20 mg salmon sperm DNA. The filters were washed twice for 20 min at room temperature with 2× SSC, 10% SDS and for 30 min at 65°C with 0.2× SSC, 10% SDS followed by autoradiography. Three positive clones were selected and plaque purified. cDNA was sequenced in both directions using a primer walking strategy. A PAC genomic library was screened using a 2 kb cDNA probe containing the ORF cDNA of *TIRC7*, and three clones representing the entire genomic cDNA of *TIRC7* and *OC116* were bidirectionally sequenced.

Northern Blot Analyses

Northern blots were prepared with 7–10 μg of total RNA as described previously (Kojima et al., 1996). $Poly(A)^+$ Northern blots containing RNA from various human tissues were purchased from Clontech. Northern blots were probed with the full-length *TIRC7* cDNA or a TIRC7-specific cDNA fragment (nt 52–391). Overnight hybridizations were performed with ^{32}P-labeled cDNA probes (10^6 cpm/μl) at 42°C in 40% formamide, 10% dextran sulfate, 4× SSC, 7 mM Tris (pH 7.6), 0.8× Denhardt's solution, 0.02 mg/ml salmon sperm DNA, and

10% SDS. Blots were washed twice in 2× SSC and 0.1% SDS for 20 min at room temperature, once at 65°C in 0.2× SSC, 0.1% SDS and autoradiographed at −80°C.

COS-7 and CHO Cell Transfection

The full-length *TIRC7* ORF was cloned upstream of a c-*myc* epitope sequence to create a fusion protein construct in a mammalian expression vector (Promega). Transient transfection of COS-7 cells and stable transfection of CHO cells was performed by lipofectamine transfection method as described (Schülein et al., 1996). An anti-c-*myc* antibody (InVitrogen) was used to detect the protein.

Western Blot Analysis and Membrane Preparations

PBLs were lysed and collected in PBS, 0.5 mM PMSF, 3.2 μg/ml trypsine inhibitor, 1.4 μg/ml aprotinin, and 0.5 mM benzamidine. Membrane preparations were obtained by centrifugation of the lysates for 1 hr at 4°C, 100,000 × g, twice. Lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Filters were blocked for 1 hr with Blotto (20 mM Tris–HCl [pH 7.5], 0.15 mM NaCl, 5% dried milk powder, 1% Triton X-100). Anti-TIRC7 antibodies were added (dilution 1:200) and incubated for 2 hr at room temperature. For peptide blockade experiments peptides were used in 1:200 dilutions and incubated with the respective antibody for 30 min at 4°C. Filters were then washed four times (15 min each) with Blotto and then incubated with alkaline phosphatase conjugated anti-rabbit IgG (1:4000 in Blotto) for 1 hr at room temperature. Filters were then washed four times with Blotto (10 min each), two times with Blotto without dried milk powder (10 min each), and once for 5 min with 10 mM Tris–HCl (pH 9.5). Filters were incubated with staining solution (0.56 mM BCIP, 0.48 mM MBT in 10 mM Tris–HCl [pH 9.5]) until bands became visible. Specificity of antibody binding was proofed by blocking with 200-fold excess of the respective peptide.

Confocal Microscopy and Flow Cytometry

Confocal microscopy studies were performed as described in Oksche et al., 1998. Briefly, cells were washed in KRH, fixed with 100 mM cocodylat, 100 mM sucrose, 10% PFA saline buffer for 30 min at room temperature and permeabilized with 0.1% Triton in PBS for 3 min at room temperature. T cells were coincubated with an anti-TIRC7 antibody (dilution 1:500) for 45 min at room temperature, blocked with 5% goat serum for 20 min at 37°C, and washed 3 times in PBS. Anti-rabbit Cy3 (Dianova, Hamburg) conjugated secondary antibody was used in 1:200 dilutions. Specific blockade of the antibody was performed using 2500-fold excess of the respective peptide. A TIRC7 nonrelated anti-rabbit IgG was used in control experiments. Flow-cytometric studies were performed as described in Waldrop et al., 1997. CD3, CD4, and CD8 rat antibodies were purchased from Pharmingen.

Preparation of Purified Peptides and Polyclonal Antibodies

Antigenic nontransmembrane regions of TIRC7 were identified using PSORT and PC-GENE and used to design short peptide sequences. Purified synthetic peptides (P1–P7) (Laboratories of Henklein, Berlin, Germany) were used for immunization of rabbits (Seramun, Berlin, Germany). Animals were boostered after three and six weeks. A total of 14 polyclonal antibodies were prepared against 7 different peptides. The pooled antisera were purified by affinity chromatography after binding of peptide to BSA. All antibodies were tested by ELISA with their respective peptides.

MLR Suppression by Anti-TIRC7 Antibodies, Cytokine Detection, and Quantitative RT-PCR

For proliferation assays, responder PBLs were plated in the presence of an equal number of irradiated-stimulator cells (total of 2×10^6 cells/ml) with either media alone, antibodies or control serum into each well of a round-bottomed 96-well microtiter plate in a final culture volume of 200 μl. Anti-TIRC7 antibodies were added in 1:500 dilutions to MLR. The plates were incubated at 37°C, 5% CO_2 and pulsed for the final 18 hr of the culture with 1 μCi [^{3}H]-thymidine (ICN Biochemicals) per well. All plates were harvested and counted on a Betaplate liquid scintillation counter. Counts were represented as the mean cpm of quadruplicate wells harvested at 72 hr following the 18 hr pulse. Peptide blocking experiments were performed by coincubation of the antibody with the appropriate peptide for 30 min at 4°C prior to initiating the MLR. The cytokine expression for IL-2, IL-4, and interferon-γ were detected in culture supernatants by commercial ELISA kits for IL-2 (Laboserv-/Medgenix/German provider of Biosource, Ratingen, Germany), IL-4-ultrasensitive (Laboserv), and IFN-γ (Laboserv).

MLR Suppression by TIRC7 Protein

Two micrograms of *TIRC7* or control cDNAs was translated in an in vitro translation TNT lysate system (Promega) containing ^{35}S-methionine (ICN). The product was visualized by SDS-page (11%) and autoradiography. For MLR inhibition experiments the in vitro translation mixtures were suspended in 500 μl PBS and dialyzed against PBS for 24 hr. For protein inhibition studies using either TIRC7 protein or two TIRC7 unrelated seven transmembrane proteins (Rantes receptor CCR1 and Leukotriene B4 receptor, kindly provided by A.D. Luster), or a TIRC7 unrelated nonmembrane protein (not published) in a concentration of 70 (c1), 50 (c2), and 30 (c3) ng/ml, respectively, of the dialyzed in vitro–translated proteins were added to MLRs. Inhibition was measured by ^{3}H-thymidine incorporation.

Renal Transplantation and Histology of Kidney Allografts

Male inbred rats 200–250 g (Harlan Winkelmann, Germany) were used throughout the experiment. Wistar Furth rats (WF, RT1^u) were grafted into bilaterally nephrectomized Lewis rats (LEW, RT1^l) using microsurgical techniques; ischemic time was 30 ± 5 min. Anti-TIRC7 Ab73 and the control antibodies (preimmune serum, and Ab77) were injected 6 times at a dose of 0.5 ml/injection.

Cryostat sections were fixed in formalin. The fixed tissue was paraffin embedded, and tissue sections were stained with hematoxylin and eosin.

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GenBank Accession Number

The *TIRC7* cDNA sequence has been deposited in GenBank under the accession number AF025374.